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A novel analytical probe binding to a potential carcinogenic factor of *N*-glycolylneuraminic acid by SELEX

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ABSTRACT

N-glycolylneuraminic acid (Neu5Gc) is an abundant sialic acid in many mammals and is present in the glycoconjugates of most deuterostome animals. Neu5Gc also occurs in fresh samples of human tumors and fetuses. However, very little is known about the expression level and biologic functions of Neu5Gc due to the limitations of available analytical probes for detection methods. In this study, we first report the development of aptamers specific to Neu5Gc screened by Systematic Evolution of Ligands by Exponential Enrichment (SELEX). After 15 selection rounds, cloning, sequencing and enzyme-linked immuno sorbent assay (ELISA) analysis, 6 different selected aptamers showed specificity for Neu5Gc. Among these 6 aptamers, N8 showed the best half maximal inhibitory concentration (IC_{50}) value (127 ng mL^{-1}) and had a relatively high affinity constant ($Ka = 6.68 \times 10^9\ M^{-1}$). The aptamers selected in this study will provide a novel analytical probe for the development of a biosensor to detect Neu5Gc in tissues and sera from patients with tumors as well as to detect Neu5Gc in animal-derived foods. In addition, the successful aptamer candidate can solve the problem that antibody is difficult to prepare in immunological assays. Thus, the discovery of novel aptamers specific for Neu5Gc is important for developing new methods of detecting Neu5Gc for the diagnosis and prevention of cancer as well as food poisoning.

1. Introduction

N-glycolylneuraminic acid (Neu5Gc) is an acidic 9-carbon sugar found primarily in deuterostome-lineage animals (Kawano et al., 1994; Chen and Varki, 2010; Varki, 1992). It plays an important role in cell-cell recognition and adherence, inflammation, immune response, and transformation of tumor cells (Yamakawa et al., 2007; Hedlund et al., 2007). However, Neu5Gc is not present in the tissues of chickens or healthy humans. The human deficiency of Neu5Gc is explained by an inactivating mutation in the gene encoding CMP-*N*-acetylneuraminic acid (Neu5Ac); this mutation is a 92-bp deletion corresponding to the loss of a single human exon homologous to exon 6 of the mouse hydroxylase gene, which was discovered after sequencing the entire corresponding region of the human genome (Muchmore et al., 1998; Varki, 2001, 2009; Malykh et al., 1998; Chou et al., 1998). However, the use of various immunological and chemical techniques has suggested that expression of Neu5Gc can be detected in some visceral cancers, such as liver, gastric and colorectal cancers (Morito et al., 1982; Devine et al., 1991; Nasonkin and Koliatsos, 2006).

Glycoconjugate-bound Neu5Gc is a common component of animal-based dietary sources, particularly in most types of red meat. Neu5Gc might be released by the action of sialidases present in the gut and could be detected in the human body following consumption and digestion of organisms that normally express Neu5Gc (Diaz et al., 2009; Hirabayashi et al., 1987; Taylor et al., 2010). However, the mechanisms of sialic acid resorption, its distribution throughout the body, its uptake by cells, and expression of its potential precursor, *N*-glycolylmannosamine (ManNGc), are not clearly known. It is reported that cultured human cells are capable of taking up free sialic acid from the medium and incorporating it into glycoconjugates in vitro. Thus, the possibility that Neu5Gc-containing glycoconjugates are taken up directly from the diet and to what extent dietary Neu5Gc-containing gangliosides can become conjugated to the sialic acids of human tissues remains to be investigated.

Immunodiagnosis of tumors is mainly based on a specific reaction between tumor antigen and antibody. As Neu5Gc is endogenously expressed in mammals, mice do not react strongly to Neu5Gc antigen, which is necessary for the preparation of monoclonal antibodies. The human anti-Neu5Gc antibodies are complex, polyclonal, and variable amongst individuals, and the

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Neu5Gc antigen can be of exogenous or endogenous origin. Indeed, both polyclonal and monoclonal antibodies against Neu5Gc have been raised in chickens and used to detect this sialic acid in tissue sections and lipid extracts from human tumors and fetuses. Although Neu5Gc can induce chickens to generate IgG or IgY immunoglobulins, the polyclonal antibody has a poor specificity. Therefore, the preparation of a specific antigenic probe is a bottleneck for development of accurate immunodiagnostics.

As an alternative to antibodies, aptamers have shown promising applications in diagnostics and therapeutics. Aptamers are ssDNA, RNA, or modified nucleic acids. They are screened from a chemically-synthesized nucleic acid combinatorial library by an in vitro selection process called "systematic evolution of ligands by exponential enrichment" (SELEX) (Tuerk and Gold, 1990; Jayasena, 1999). Because of their unique three-dimensional structures, aptamers can bind selectively and specifically to peptides, proteins, and pathogenic targets by hydrogen bonding, Van der Waals forces, hydrophobic effects and other mechanisms (Tuerk et al., 1992; Sassanfar and Szostak, 1993; Wilson et al., 1998; Macaya et al., 1993; Drolet et al., 1999). In contrast to antibodies, aptamers are non-immunogenic, consistent, animal-friendly, and small (5—15 kDa), and aptamers can show favorable target-to-objection ratios in a short time at a relatively low cost, allowing better batch-to-batch reproducibility and easier incorporation of chemical modifications. Aptamers, which are synthetic molecules, have emerged as being potentially applicable to the diagnosis and treatment of cancer by inhibiting tumor angiogenesis, blocking signal transduction, inhibiting tumor cell proliferation, promoting tumor cell apoptosis, inhibiting tumor metastasis and as immunotherapy treatments (Bock et al., 1992).

In this study, we report the first development of DNA aptamers that are able to bind Neu5Gc with high affinity and selectivity. The results presented in this report provide the first step in the development of an affordable, sensitive and high-throughput assay for establishing relatively easy and quick laboratory detection methods for Neu5Gc.

2. Materials and methods

2.1. Reagents and materials

The synthetic random DNA library, primers and biotin-labeled primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Company (Shanghai, China). Bovine serum albumin (BSA) was purchased from Beijing Dingguo Biotechnology Development Centre (Beijing, China). *N*-hydroxysuccinimide, *N*,*N*-dicyclohexylcarbodiimide (DCC), and *N*,*N*-dimethylformamide (*N*,*N*-DMF) were purchased from Sigma (CA, USA). Streptavidin conjugated to horseradish peroxidase was purchased from Beijing Biosynthesis Biotechnology Co., LTD (Beijing, China). 96-well ELISA plates were purchased from Costar (NY, USA). Deionized and pyrogen-free water was obtained from a Milli-Q water purification system (Millipore, USA). Taq DNA polymerase and PMD-18 T were purchased from TaKaRa (Dalian, China). Other reagents were of analytical purity.

2.2. Preparation of Neu5Gc conjugates as a screening target

For screening, Neu5Gc was conjugated to carrier protein and coated into the wells of a 96-well plate by the active ester method (Lu et al., 2012). Briefly, the carboxyl group of Neu5Gc was reacted with *N*-hydroxysuccinimide by using DCC to generate active ester derivatives, which were then reacted with the amino terminus of bovine serum albumin (BSA) to form conjugates. The successfully prepared conjugates were then coated in a microplate as a target substance for selection.

2.3. Random library and primers

The random DNA library contained a central randomized sequence of 40 nucleotides (N_{40}) flanked by a 21-nucleotide constant region (5′-CATCTGCAGTGTGGCACCATG-N_{40}-CGTGCTGAG CGTGAATTCGC-3′). A 5′ (+) strand primer up (5′-CATCTG-CAGTGTGGCACCATG-3′) and a 3′ (-) strand primer down (5′-GCGAATTCACGCTCAGCACG-3′) were used to synthesize double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) by regular PCR or asymmetric PCR, respectively. A 5′ (+) strand biotinylated primer was used for assessing binding activity.

2.4. In vitro selection of aptamers for Neu5Gc

For the first round of SELEX, 125 μL of the synthetic ssDNA library and 75 μL of deionized water were added to 250 μL of binding buffer. Then, the ssDNA library was denatured by heating at 96 °C for 10 min and cooled immediately on ice for 10 min while the coated microplate was washed 3 times with washing buffer. The denatured ssDNA library (100 μL) was added to the coated microplate and incubated at 25 °C for 12 h. Following washing, the Neu5Gc-bound ssDNAs were eluted with 100 μL of elution buffer at 80 °C for 15 min. The pooled eluted oligonucleotides were precipitated by ethanol precipitation. To avoid enrichment of nonspecific ssDNAs during the process, counterselection was performed as follows: negative selection with uncoated plate after 4 and 8 rounds to remove nonspecific ssDNA binding followed by counterselection with microtiter plates coated with BSA only (no Neu5Gc).

2.5. Ethanol precipitation of ssDNA

The ssDNA was extracted with an equal volume of phenol/chloroform/isoamylol (25/24/1) by centrifuging at 12,000 rpm for 4 min; then, 5 μL of 2.5 mol L^{-1} sodium acetate and 2.5 volumes of 100% ice-cold ethanol were added to the supernatant. The solution was incubated at −20 °C for 1 h to ensure complete precipitation and then centrifuged at 10,000 rpm for 15 min at 4 °C to collect the precipitated ssDNA. The ssDNA pellet was washed in 75% ice-cold ethanol and then centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, leaving the pelleted ssDNA at the bottom of the vial. The pellet was dried at 37 °C and resuspended in 20 μL of TE buffer. The precipitated DNA was amplified by PCR followed by asymmetric PCR for the next round of selection.

2.6. PCR and asymmetric PCR

After precipitation, the library was subjected to PCR for 25 cycles in a 25 μL mixture containing 1 μL of DNA template, 10 μmol of each primer up and down 2.5 mmol L^{-1} of each dNTPs, 15 mmol L^{-1} $MgCl_2$, and 2.5 U of Taq DNA polymerase. To generate single-stranded aptamers, the resulting amplicons were subjected to 25 cycles of asymmetric PCR containing 1 μL of DNA template, 10 μmol of primer and 1.25 μmol of primer down-1, 2.5 mmol L^{-1} of each dNTPs, 15 mmol L^{-1} $MgCl_2$, and 2.5 U of Taq DNA polymerase.

2.7. ABS-ELISA assay

Sandwich enzyme-linked immunosorbent assay using avidin—biotin (AB-ELISA) was developed to assess the enrichment of the library for the target binding as well as to determine which rounds of eluted ssDNA were to be used for clone sequencing. To coat the microtiter plates, they were incubated for 2 h at 37 °C with 100 μL $well^{-1}$ of Neu5Gc-BSA (1 μg mL^{-1}) in sodium carbonate buffer, pH 9.6. Then, the plates were blocked with PBS (Phosphate Buffered Saline) containing 5% skim milk powder and kept covered at 37 °C

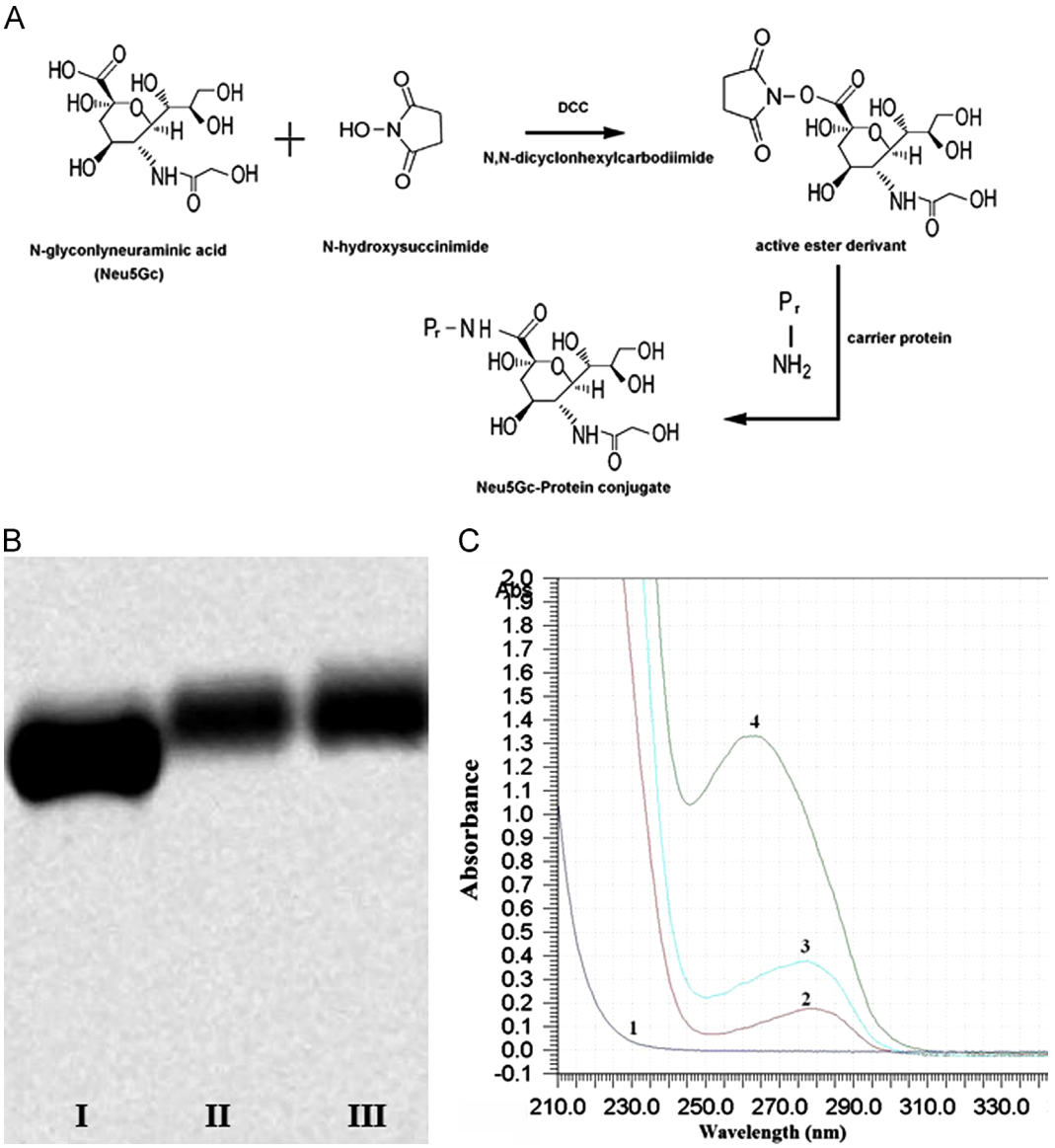


Fig. 1. (A): Reaction scheme of synthesis for Neu5Gc-protein conjugates. Pr—NH_2: carrier protein. (B) Agarose gel electrophoresis: lane I:Neu5Gc-BSA; lane II: reagent-treated BSA; lane III: BSA. (C) UV spectra for the conjugates: "line 1" is the UV spectra of Neu5Gc; "line 2" is the UV spectra of BSA; "line 3" is the UV spectra of Neu5Gc-BSA conjugates; "line 4" is the UV spectra of reagent-treated BSA.

for 1 h. Each round of the biotin-labeled Neu5Gc aptamer population (PCR products) was diluted in binding buffer at 1:100, denatured for 10 min at 96 °C, and cooled immediately on ice. Next, 100 μL of the solution was added to each well; the plate was incubated at 37 °C for 1 h. Afterward, 100 μL of a 1:1000 dilution of HRP-streptavidin was added to the individual wells. After a 1 h incubation at 37 °C on a shaking platform, the peroxidase reaction was developed using *o*-phenylenediamine dihydrochloride and hydrogen peroxide (OPD $+H_2O_2$). OD_{492} nm values were determined using a microplate reader from BioTex, Epoch. Following each incubation step, a washing step was performed with 200 μL of washing buffer to remove unreacted molecules.

2.8. Clone sequencing and analysis of the selected aptamers

After 15 rounds of selection, the highest affinity pool was PCR amplified with unlabeled primer up and down and cloned into *Escherichia coli* DH5a using the PMD-18T vector system (TAKARA biotechnology Co. Ltd.) to generate dsDNA. Separate colonies were selected at random, and their sequences were determined by Sangon Biotech (Shanghai) Co., Ltd. Sequence alignments were performed using GENEDOC software, which allowed analysis of conserved and consensus regions. Prediction of the lowest free energy shapes and secondary structure analyses of the sequences were executed by using the free-energy minimization algorithm via the internet tool m-fold.

2.9. The affinity test of aptamers

After plasmid extraction and PCR, the concentration of each aptamer was tested by measuring the OD value in accordance with the process in 2.7. The affinity of aptamers was tested using indirect ELISA. The coating antigen Neu5Gc-BSA was added into a microtiter plate at 8, 4, 2 and 1 μg mL^{-1} and incubated at 4 °C overnight. The concentration of aptamers was diluted from 5 ng mL^{-1}, and the dilution factor was 1:2. The other steps were the same with 2.7.

2.10. Competitive activity by direct competitive ELISA

A direct competitive ELISA was performed as follows: 100 μL of the coating antigen Neu5Gc-BSA diluted in the coating buffer at 1 μg mL^{-1} was added into a microtiter plate and incubated at 4 °C overnight. Plates were washed three times using 220 μL $well^{-1}$ of washing buffer followed by incubation with 50 μL $well^{-1}$ of Neu5Gc at 0, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ng mL^{-1} (for inhibition assay) in 0.01 mol L^{-1} PBS together with 50 μL $well^{-1}$ ssDNA (5 ng mL^{-1} in the dilution buffer solution) for 1 h at 37 °C. After washing three times, 100 μL of HRP-streptavidin was added to each well and incubated for 1 h at 37 °C. The plates were washed again, and 100 μL $well^{-1}$ of OPD solution was added. After incubation for 10 min at 37 °C, the reaction was stopped by adding 50 μL of 2 mol $L^{-1}H_2SO_4$ $well^{-1}$. The absorbance was measured at 492 nm and recorded.

2.11. Accuracy and precision evaluation

The accuracy and precision of the selected aptamers were also evaluated. According to the value of the calibration curve, 0, 10 and 100 ng mL^{-1} Neu5Gc solutions were chosen to test the accuracy and precision. The experiments performed for analysis were repeated three times a day and continued for four days.

2.12. Statistical analysis

All date presented were the average values from more than three replicate determinations. The results were expressed as mean values with a standard deviation (SD). The statistical analysis of date was performed using SPSS 18.0 for windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Conjugation of Neu5Gc and immobilization on microtiter plates

The active ester of Neu5Gc was prepared by two general approaches: activation of the acid by routine procedures followed by reaction with the hydroxyl compound HOR2, which may be present during the activation or added afterwards; and transesterification of the oxy moiety of OR2 from the carbonic acid ester to the Neu5Gc. A schematic of this process is shown in Fig. 1A.

The Neu5Gc-BSA conjugates were analyzed by agarose gel electrophoresis and UV spectrometry. As shown in Fig. 1B, the Neu5Gc-BSA conjugates (line I) migrate more slowly than the reagent-treated BSA (line II) and the BSA (line III). Each of these different substances has different ultraviolet absorptions. If the structure of the molecule is changed, the ultraviolet absorption will also change accordingly. Thus, the value of absorbance or the absorption wavelength of the carrier protein BSA will be altered when coupled to Neu5Gc. As shown in Fig. 1C, when the coupling reagent is added to the BSA solution, the absorption wavelength of BSA changes from approximately 280 nm (line 2) to 260 nm (line 4); accordingly, when the coupling reagent activates the reaction to conjugate Neu5Gc to BSA, the absorption wavelength of Neu5Gc-BSA changes from approximately 280 nm (line 2) to 275 nm (line 3). The results indicate that Neu5Gc was conjugated to BSA successfully and can be used for binding antigen.

3.2. In vitro selection of Neu5Gc-binding aptamers

In this study, we used asymmetric PCR to preferentially amplify one strand of the dsDNA to generate ssDNA. Because the G+C content of the engineered primer sequence is not high, we initially used a high annealing temperature to avoid nonspecific binding. Annealing temperature can have a tremendous impact on the purity of the product, with a low annealing temperature yielding higher molecular weight products, which might be the result of high complexity in the ssDNA library. The fixed and random areas of oligonucleotide region can cause renaturation at the low annealing temperature.

The purity of ssDNA must be high because contamination with double-stranded components will lead to automatic renaturation within the dsDNA in the library with increasing screening rounds, forming a stable double helical structure. Conjugation between the ssDNA and the target molecules will cause a loss of aptamers with high specificity. We therefore adjusted the proportion of non-restricted primers to restricted primers to increase the concentration of specific ssDNA produced.

During each round of selection, the ssDNAs bound by Neu5Gc were eluted by salt and heat denaturation, allowing recovery of strongly bound Neu5Gc aptamers. After denaturing at 96 °C and cooling on ice, the binding affinity of the aptamers increases as a result of the ssDNA being more likely to form secondary structures at room temperature. The amount of ssDNA bound to Neu5Gc was enriched as the selection rounds progressed. The utilization of microplates as the binding surface may affect the natural configuration or cover up the target molecule and binding epitope, affecting the free movement of target molecules. Additionally, BSA has a much larger molecular structure than the target molecule. These factors are not conducive to enrichment of the nucleotide sequence from the library. However, microplate screening is relatively simple and comparatively economical. In the presence of metal cations, ssDNA can naturally form pseudoknots, bulge loops, G-quartets and other secondary structures, which may recognize the binding sites on the target material and form specific, high affinity interactions.

During the selection process, the aptamers with strong affinity may be lost (e.g., use of conventional phenol—chloroform extraction and ethanol precipitation), resulting in low recovery efficiency. Although this study has technical limitations, these issues could be solved by studying the mechanism more empirically. However, as a preliminary study, aptamers that bind specifically and preferentially to Neu5Gc were found and characterized.

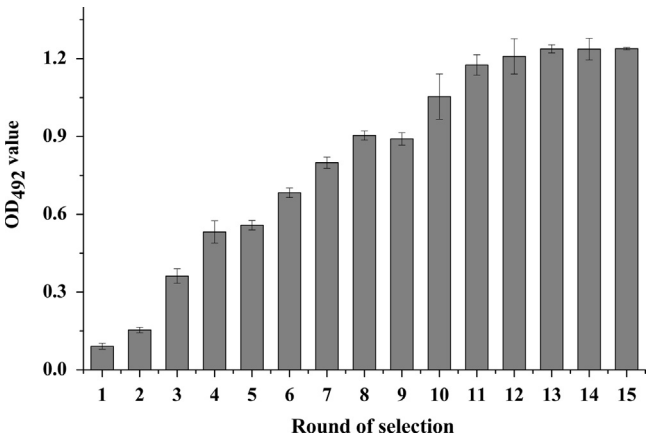
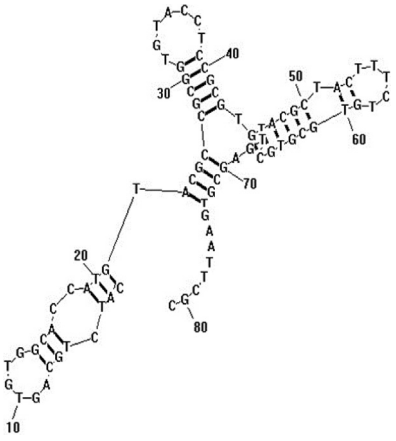
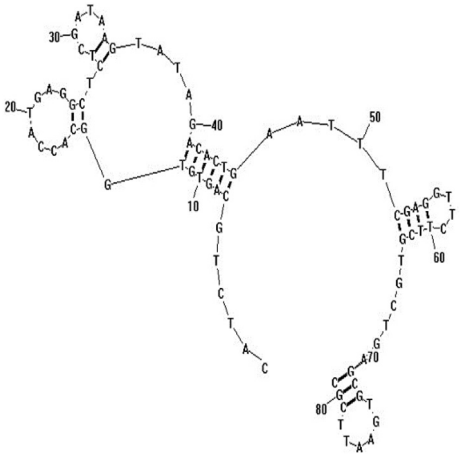
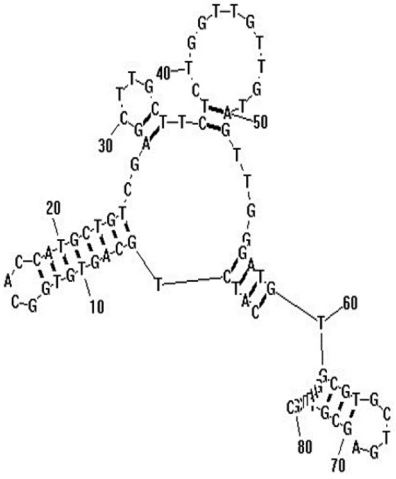
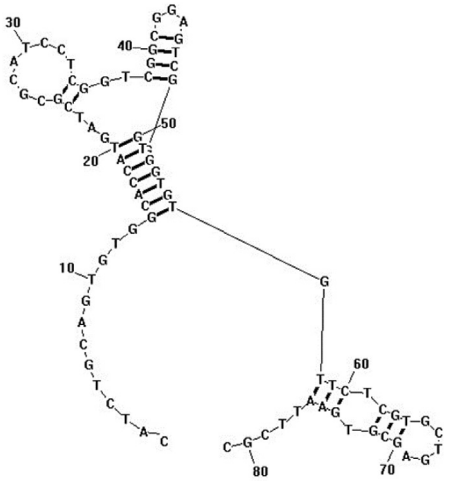
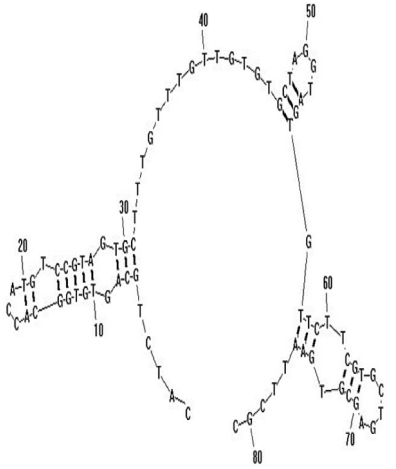
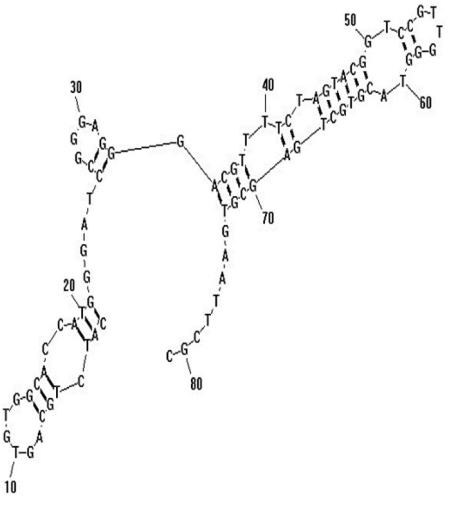


Fig. 2. The result of ABS-ELISA, test the OD value of each round of ssDNA pool. The OD values of each round were 0.091 ± 0.01, 0.154 ± 0.01, 0.362 ± 0.03, 0.532 ± 0.04, 0.558 ± 0.02, 0.683 ± 0.01, 0.799 ± 0.02, 0.904 ± 0.02, 0.891 ± 0.02, 1.054 ± 0.09, 1.176 ± 0.04, 1.209 ± 0.07, 1.238 ± 0.02, 1.237 ± 0.04 and 1.239 ± 0.00 (SD < 0.01). Counter selection was performed using uncoated plate and BSA coated plate after round 4 and 8. Date are expressed as the mean ± SD (n=3).

3.3. The result of SELEX

The ABS-ELISA shows that the ssDNA library binding to Neu5Gc increases as a function of the number of screening rounds and increased experimental conditions (e.g., temperature, pH, and incubation time). As shown in Fig. 2, the OD value from the thirteenth round was approximately 13.6 times the value from the first round, which demonstrates that the sequences of low binding affinity are gradually phased out leading to enrichment for high affinity aptamers in the majority of the ssDNA library. There is no significant increase in the OD value after 13 rounds, indicating that the assay has reached its plateau and that the binding affinity has

Table 1
The secondary structures of the 6 aptamers.

Aptamers	N8	N14
Secondary structure		
Aptamers	N15	N17
Secondary structure		
Aptamers	N20	N32
Secondary structure		

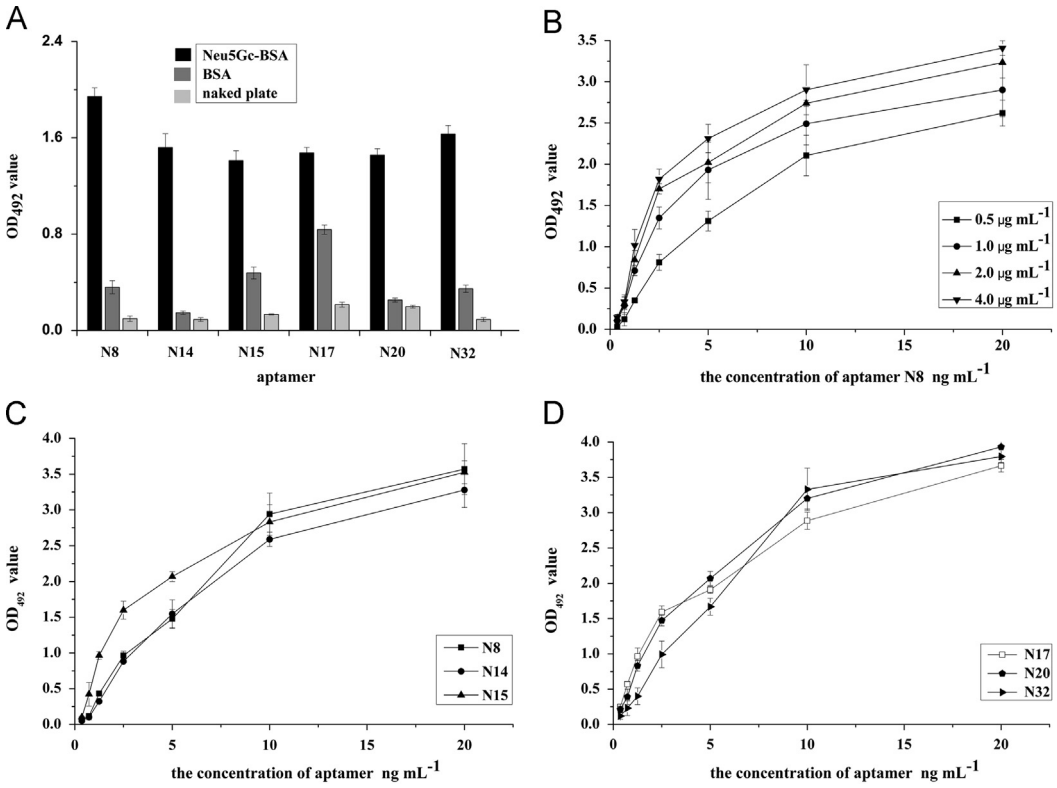


Fig. 3. Affinity test: (A) Specificity tests of aptamers using uncoated plate and BSA-coated plate. The concentration of aptamers in the test was 5 ng mL^{-1}. (B) Affinity of aptamer N8. The *X*-axis and *Y*-axis of the curve were the concentration of aptamer N8 and OD value, respectively. The concentrations used for Neu5Gc-BSA were 0.5, 1, 2 and 4 μg mL^{-1}. (C) The OD value of aptamer N8, N14 and N15 in different concentrations. (D) The OD value of aptamer N17, N20 and N32 in different concentrations. Each point represents the mean ± SD from 3 determinations ($n=3$).

been saturated. The OD value in the fifth (0.558 ± 0.02) and ninth (0.891 ± 0.02) SELEX rounds did not increase obviously, most likely due to the presence of some aptamers in the ssDNA pool that bind to the uncoated microplate or BSA. This result indicated that the ssDNA aptamers binding nonspecifically to the microplate or BSA were being discarded.

3.4. Sequence analysis of the selected aptamers

36 clones were randomly selected to be sequenced. DNAMAN was used to analyze the homogeneous sequences from the 36 clones, which could be divided into 13 classes based on common primary sequence motifs.

Similarly, the prediction of secondary structure reveals that these aptamers were mainly stem-loop or hairpin. As shown in Table 1. In some sequences, the aptamers are most likely forming false knot structures that might be the result of the interlap of interspace conformation between contiguity interactions among rings. We can observe the emergence of the G-tetramer in a few sequences; this structure has special thermodynamic stability in its spatial conformation that can be embedded in the surface cracks of the G-tetramer molecules. In some sequences (N15, N17, N20 and N32) with the G—T mismatches, base mismatch often occurs in the normal double helix of base pairs between the accumulations at the turning point, easily leading to the closure of the ring.

3.5. The results of affinity tests

To determine the affinities of the 6 individual aptamers for their target, the OD values were ascertained by ABS-ELISA. The 6 aptamers showed a high affinity for Neu5Gc. As shown in Fig. 3C and D, different concentrations of aptamers were tested to determine the OD value. Aptamers were tested in a concentration range from 20 ng mL^{-1} to 0.3625 ng mL^{-1}. N14 showed a relatively lower OD value compared to other aptamers. Specificity of the selected aptamers was tested by using uncoated plates and plates coated with BSA. N8, N14, N15, N20 and N32 showed a distinct specificity for the target molecule when compared with BSA-coated or uncoated plates, as shown in Fig. 3A. However, N17 showed a higher affinity to BSA (OD = 0.837 ± 0.04), indicating that it most likely binds on the molecular connection between Neu5Gc and BSA. Additionally, aptamer N8 showed a high affinity for Neu5Gc as a target molecule (OD = 1.943 ± 0.07) as compared to other aptamers.

Aptamer N8 was tested for its ability to bind Neu5Gc. The calibration curve is shown in Fig. 3B. The OD_{max} is set at the top of the curve. The binding affinity of aptamers was calculated using the following equation: $Ka=(n-1)/(n[Ap']t-[Ap]t)$. In this equation, n is the concentration ratio of plates coated with two different concentrations of Neu5Gc-BSA in one group, [Ap′] and [Ap] are the concentrations (mol L^{-1}) of aptamer corresponding to 50% of maximum absorbance values of plates coated with two different concentrations of Neu5Gc-BSA. The average is the affinity constant. The affinity constant of N8, N14, N17 and N32 were determined to be 6.68×10^{9} M^{-1}, 6.13×10^{9} M^{-1}, 4.58×10^{9} M^{-1} and 6.49×10^{9} M^{-1}, respectively, whereas the Ka values for N15 and N20 were relatively lower at 7.26×10^{8} M^{-1} and 7.33×10^{8} M^{-1} and were thus considered to have weak binding capacity.

3.6. Relative test of aptamers via dc-ELISA

To analyze the competitive binding activity by direct competitive ELISA, the aptamers were reacted with dilutions of Neu5Gc, and the mix was added to the microplate, therefore creating a competition between the immobilized Neu5Gc and the free

Neu5Gc. Different concentrations of immobilized Neu5Gc were reacted with various concentrations of aptamers. The optimal working concentrations for Neu5Gc-BSA and HRP-streptavidin were 1 μg mL^{-1} (Fig. 4A) and 1:1000, respectively. Different blocking reagents were used to acquire the lowest absorption value, with 5% skim milk being the preferable blocking agent. Aptamers were added to the reaction at a concentration of 5 ng mL^{-1}. We choose N8 to investigate in the competition assays because this aptamer showed increased sensitivity at a low concentration of aptamer. The sensitivity of aptamers was detected under optimal assay conditions and expressed by IC_{50} (50% inhibition concentration) value. The competition curves of the aptamers are shown in Fig. 4B and C, which all exhibited good linearity ($R^2 > 0.989$). When 6 aptamers were tested by direct competition ELISA, the 50% inhibition concentration (IC_{50}) of N8, N14 and N32 were calculated as 127 ng mL^{-1}, 198 ng mL^{-1} and 227 ng mL^{-1}, respectively, whereas the IC_{50} values for N15, N17 and N20 were slightly higher at 5315 ng mL^{-1}, 1189 ng mL^{-1} and 1254 ng mL^{-1}, respectively. The IC_{10} (10% inhibition concentration) value for N8, N14, N15, N17, N20 and N32 were 5 ng mL^{-1}, 7 ng mL^{-1}, 17 ng mL^{-1}, 6 ng mL^{-1}, 9 ng mL^{-1}, and 6 ng mL^{-1}, respectively. These 6 aptamers were suitable for direct competitive assays because addition of free Neu5Gc allowed the displacement of the bound aptamer from the immobilized Neu5Gc, which differs from the anti-Neu5Gc antibodies, which show a higher affinity to Neu5Gc-BSA than to free Neu5Gc.

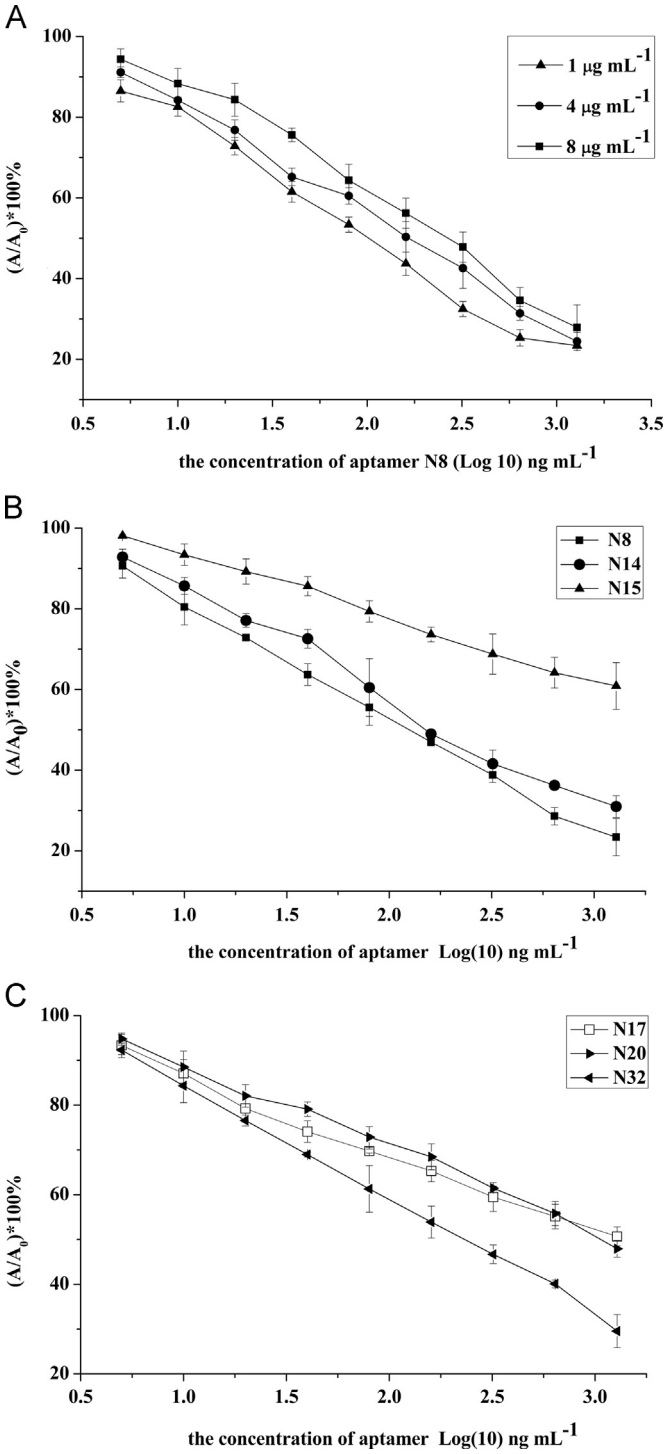


Fig. 4. Direct competition ELISA: (A) Calibration curves for Neu5Gc by dc-ELISA, using different dilutions of aptamer N8. The concentrations for Neu5Gc-BSA are 1, 4 and 8 μg mL^{-1}. (B) Competition curves of aptamers N8, N14 and N15. The IC_{90} value for N8, N14 and N15 were determined to be 3.32 μg mL^{-1}, 5.89 μg mL^{-1} and 1658 μg mL^{-1}, respectively. (C) Competition curves of aptamers N17, N20 and N32. The IC_{90} value for N17, N20 and N32 were 232 μg mL^{-1}, 173 μg mL^{-1} and 8.56 μg mL^{-1}, respectively. Each date point represents the mean ± SD of 3 determinations.

3.7. Accuracy and precision of the aptamers

Series of Neu5Gc standard solutions (0, 10 and 100 ng mL^{-1}) were measured to determine the accuracy and precision of the aptamers. Inter-assay variation was obtained based on the three times' measurements within one day and the intra-assay variation was calculated by the results of four days. As shown in Table 2, the inter-assay variation (0.22%—5.85%) and the intra-assay variation (< 13%) were both acceptable. It was indicated that the selected aptamers were stable and credible for determining the concentration of Neu5Gc.

4. Conclusions

In this study, we report the development of aptamers specific for Neu5Gc for the first time. The 6 different aptamers selected for affinity for Neu5Gc were investigated by using ELISA and direct competitive ELISA. Of these 6 aptamers, N8 showed the best IC_{50} value (127 ng mL^{-1}) and had a relatively high affinity constant (Ka = 6.68 × 10^9 M^{-1}). Due to its high sensitivity and specificity, the

Table 2
Intra-assay and inter-assay variation coefficients.

Aptamer	Concentration (ng mL^{-1})	Inter-assay (n=3)		Intra-assay (n=4)	
		Mean ± SD (X ± SD)	CV/%	Mean ± SD (X ± SD)	CV/%
N8	0	0.913 ± 0.013	1.43	0.978 ± 0.030	3.09
	10	0.814 ± 0.009	1.16	0.868 ± 0.060	6.89
	100	0.524 ± 0.013	2.48	0.565 ± 0.040	7.16
N14	0	0.995 ± 0.015	1.51	1.079 ± 0.062	5.73
	10	0.896 ± 0.021	2.37	0.898 ± 0.028	3.07
	100	0.556 ± 0.011	1.98	0.624 ± 0.480	7.69
N15	0	1.153 ± 0.004	0.30	1.158 ± 0.70	6.00
	10	1.058 ± 0.062	5.85	1.049 ± 0.080	7.65
	100	0.848 ± 0.016	1.90	0.856 ± 0.051	6.01
N17	0	0.945 ± 0.008	0.85	0.997 ± 0.019	1.86
	10	0.855 ± 0.007	0.81	0.886 ± 0.024	2.70
	100	0.732 ± 0.009	1.21	0.775 ± 0.038	4.94
N20	0	1.044 ± 0.014	1.30	1.037 ± 0.686	6.61
	10	0.939 ± 0.002	0.22	0.904 ± 0.062	6.81
	100	0.737 ± 0.013	1.70	0.768 ± 0.094	12.21
N32	0	1.018 ± 0.012	1.15	1.042 ± 0.079	7.55
	10	0.920 ± 0.015	1.67	0.887 ± 0.050	5.69
	100	0.671 ± 0.002	0.30	0.684 ± 0.075	11.01

aptamers selected in this study can be used as a recognition element for the development of Neu5Gc detection assays. Further work is aimed at using this aptamer to develop assays for detecting Neu5Gc in tissue and related serum from tumor patients, as well as for detecting Neu5Gc in foods that were derived from animals.

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